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REDD1, a Developmentally Regulated Transcriptional Target of p63 and p53, Links p63 to Regulation of Reactive Oxygen Species

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Summary

We identified *REDD1* as a novel transcriptional target of p53 induced following DNA damage. During embryogenesis, *REDD1* expression mirrors the tissue-specific pattern of the p53 family member p63, and *TP63* null embryos show virtually no expression of *REDD1*, which is restored in mouse embryo fibroblasts following p63 expression. In differentiating primary keratinocytes, *TP63* and *REDD1* expression are coordinately downregulated, and ectopic expression of either gene inhibits in vitro differentiation. REDD1 appears to function in the regulation of reactive oxygen species (ROS); we show that *TP63* null fibroblasts have decreased ROS levels and reduced sensitivity to oxidative stress, which are both increased following ectopic expression of either *TP63* or *REDD1*. Thus, *REDD1* encodes a shared transcriptional target that implicates ROS in the p53-dependent DNA damage response and in p63-mediated regulation of epithelial differentiation.

Introduction

The tumor suppressor gene *TP53* encodes a transcriptional activator that functions as a key nodal point for integrating cellular responses to genomic damage (reviewed in Vogelstein et al., 2000). While p53 is largely dispensable for normal development, its disruption is a common event in human cancers. The role of p53 as a guardian of genomic integrity is supported by its activation following ionizing radiation and inappropriate cellular proliferation signals, and by the development of tumors in *TP53* null mice and in humans with Li-Fraumeni Syndrome (Giaccia and Kastan, 1998). Ectopic expression of p53 triggers both cell cycle arrest and apoptosis in cultured cells. G_1 phase arrest has been linked primarily to transcriptional activation of the cyclin-dependent kinase inhibitor $p21^{Cip1}$ (Deng et al., 1995), while a number of candidate target genes have been implicated in p53-dependent apoptosis, including *Bax*, *TRAIL-DR5*, *PERP*, *Noxa*, and *APAF-1* (Miyashita and Reed, 1995; Attardi et al., 2000; Oda et al., 2000; Takimoto and El-Deiry, 2000; Moroni et al., 2001). Additional p53 transcriptional targets are thought to contribute to apoptosis by regulating cellular redox status following DNA damage (Polyak et al., 1997; Li et al., 1999). Still others contribute to diverse cellular processes such as DNA repair, genome stability, angiogenesis, and regulation of p53 turnover (El-Deiry, 1998; Tanaka et al., 2000).

The discovery of two *TP53*-related genes in mammals, *TP63* and *TP73*, has provided new insight into the evolutionary function of this gene family (Irwin and Kaelin, 2001). *TP63* appears to be the most ancient family member, and it is most closely related to the single gene present in *Drosophila* (Yang et al., 1998). In contrast to *TP53*, neither *TP63* nor *TP73* appears to be targeted by mutations in human cancer, although some links to the DNA damage response pathway have been demonstrated (Gong et al., 1999; Yuan et al., 1999; Flores et al., 2002). However, both p73 and p63 play essential roles in development, the former being essential for brain development, and the latter for formation of epithelial structures, including skin, limbs, hair follicles, and mammary glands (Mills et al., 1999; Yang et al., 1999, 2000). Notably, p63 is expressed in primitive epithelial precursors, and *TP63* null mice display an apparent depletion of epithelial stem cell reserve, leading to severe developmental defects. While p63-mediated regulation of cell cycle progression and apoptosis has been demonstrated in cultured cells, the mechanism by which this transcription factor modulates cellular differentiation is unknown (Yang et al., 1998; Dohn et al., 2001).

A fundamental question regarding p53 family members is whether their diverse physiological functions reflect tissue-specific differences in expression, or regulation of distinct sets of transcriptional targets. The DNA binding domain of p63 is highly homologous to that of p53, and ectopic overexpression of p63 leads to transcriptional activation of a subset of known p53 targets (Dohn et al., 2001). However, it is unclear whether these genes are physiologically regulated by p63. A further level of complexity stems from the multiple splicing variants derived from the *TP63* gene. The N-terminal splice variant that is most similar to p53, TAp63γ, mediates transcriptional activation in promoter-reporter assays, but it represents a very small subset of the cellular p63 transcript (Yang et al., 1998). The most abundant isoform, ΔNp63α, lacks the *trans*-activational domain (Parsa et al., 1999). This truncated form has been postulated to function as a dominant-negative protein, capable of inhibiting expression of p63 target genes as well as targets of p53 and p73, through its heterotypic interaction with these family members (Yang et al., 1998; Gaiddon et al., 2001). As such, it is uncertain whether physiological expression of p63 during normal development leads to induction or repression of specific target

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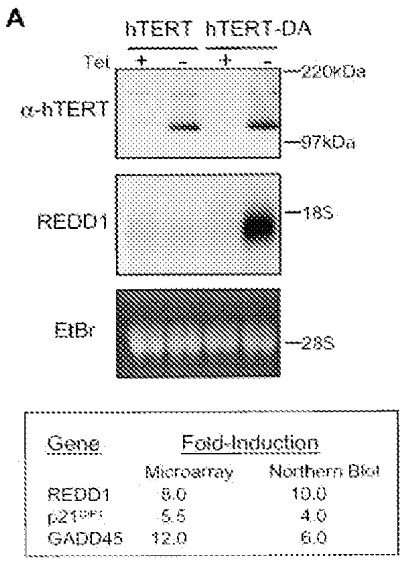

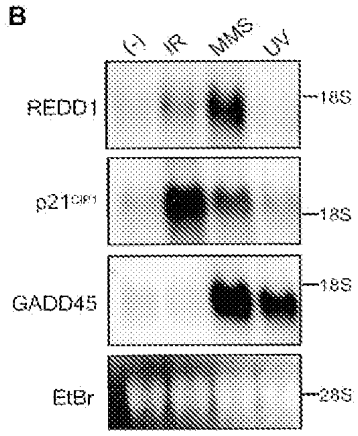

Figure 1. Induction of REDD1 following Multiple Forms of DNA Damage

(A) Induction of *REDD1* following expression of mutant hTERT (hTERT-DA). Western blot analysis of lysates from cells with tetracycline (tet)-repressible expression of wild-type hTERT or hTERT-DA, demonstrating inducible expression of the transfected construct (top panel). Induction of *REDD1* mRNA 24 hr following tetracycline withdrawal is shown by Northern blot (middle panel), with loading control (EtBr). The table summarizes the results of microarray analysis and Northern blot validation by densitometry for the three most highly induced sequences following hTERT-DA expression.

(B) Northern blot analysis of primary diploid fibroblasts, either untreated (−), or 12 hr following 10 Gy ionizing radiation (IR), 100 μg/ml methyl methane sulphonate (MMS), or 20 J/m^2 ultraviolet radiation (UV). The same blot was sequentially probed with *REDD1*, *p21^{Cip1}*, and *GADD45* (EtBr, loading control).

genes. In either case, the functional properties of p53- and p63-responsive gene products provide important insight into the cellular pathways regulated by these transcription factors.

Here we report the identification of a novel gene expressed broadly in adult cells and transcriptionally regulated by p53 following ionizing radiation, yet expressed in a tissue-specific manner and regulated by p63 during embryonic development and epidermal differentiation. This gene, which we have named *REDD1* (for *r*egulated in *d*evelopment and DNA *d*amage responses), provides a paradigm for the functional relationship between p53 and p63. REDD1 appears to function in the regulation of cellular ROS, a pathway linked to both stress responses and modulation of growth factor signaling (Adler et al., 1999; Finkel, 2000; Shoshani et al., 2002). *TP63* null MEFs have low levels of endogenous *REDD1*, and they demonstrate decreased endogenous ROS and reduced sensitivity to oxidative stress. Restoration of either REDD1 or p63 expression in these cells increases ROS induction and corrects oxidative stress sensitivity; regulation of ROS contributes to the ability of REDD1 to inhibit keratinocyte differentiation. Together these observations point to a role for ROS in p63-regulated epithelial differentiation, as well as in p53-dependent stress responses.

Results

REDD1 mRNA Is Upregulated following Multiple DNA Damage Stimuli

Our initial objective was to identify genes whose expression is upregulated following telomere destabilization, then to compare these with genes induced following other DNA damage stimuli. We established tetracycline-regulated expression of the human telomerase catalytic subunit (hTERT) and a mutant with an aspartic acid to alanine substitution in the TERT enzymatic active site (hTERT-DA) (Figure 1A). The analogous mutant in yeast is catalytically inactive and provokes telomere shortening when overexpressed (Lingner et al., 1997). As predicted, inducible expression of the mutant construct led to an increased frequency of telomere fusions, accompanied by growth arrest and cell death, whereas expression of wild-type hTERT had no effect (data not shown). RNA samples were collected from cells at several time points following induction of hTERT-DA, labeled, and hybridized to oligonucleotide microarrays representing 5500 human genes and ESTs. Hybridizations were performed in duplicate, and results were verified using Northern blot analysis (Figure 1A). Only three genes were found to be reproducibly induced following expression of TERT-DA: the cyclin-dependent kinase inhibitor *p21^{CIP1}*, the DNA damage-responsive gene *GADD45*, and an EST that we have called *REDD1*.

Induction of *p21^{CIP1}* and *GADD45* following different DNA damaging agents is known to be mediated by distinct pathways (Lakin and Jackson, 1999). We therefore compared the relative induction of these transcripts with that of *REDD1* in primary human fibroblasts, using standard doses of ionizing radiation (IR), ultraviolet radiation (UV), or treatment with the DNA alkylating agent methyl methane sulphonate (MMS). Under these conditions, each gene showed a distinct pattern of induction: *GADD45* was induced primarily by MMS and UV; *p21^{Cip1}* by IR, followed by MMS; and *REDD1* by MMS, followed by IR (Figure 1B). Therefore *REDD1*, like these well-characterized DNA damage-responsive genes, is also a target of multiple pathways involved in the signaling of genomic injury.

REDD1 Is a Direct Transcriptional Target of p53

The mechanism of *p21^{Cip1}* induction by MMS is not well understood, but its induction by IR has been used to define the classical DNA damage response pathway, which involves ATM-dependent phosphorylation and stabilization of p53 (Morgan and Kastan, 1997). To determine whether the IR-mediated induction of *REDD1* is also dependent upon this pathway, we examined *REDD1* expression in human primary fibroblasts lacking either p53 or ATM. In L3819 and L4402, two independent *TP53* null fibroblast lines derived from patients with Li-

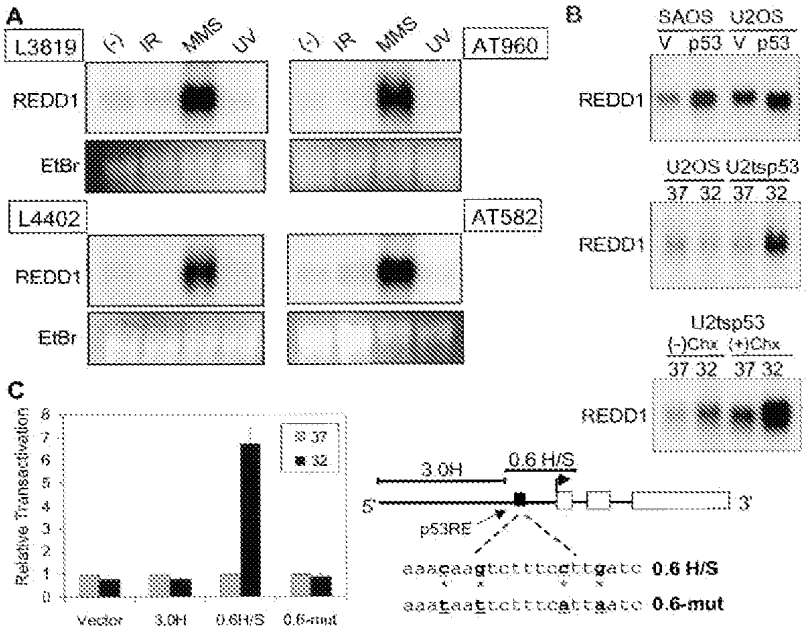

Figure 2. REDD1 Is a Transcriptional Target of p53

(A) Northern blot analysis of *TP53* null primary fibroblasts from two patients with Li-Fraumeni syndrome (L3819 and L4402) and of *ATM* null cells from two patients with ataxia telangiectasia (AT960 and AT960) following DNA damage as in Figure 1. Induction of *REDD1* by IR is abrogated, while its induction following treatment with MMS is unaltered.

(B) Northern blot demonstrating expression of endogenous *REDD1* mRNA in Saos-2 and U2OS cells, following transient transfection with a p53 expression construct (p53) or vector control (V) (top panel). Expression of endogenous *REDD1* in U2OS cells with constitutive expression of temperature-sensitive p53 (U2tsp53) is shown following growth at 37°C (primarily mutant conformation) or temperature shift to 32°C (wild-type p53). No effect is seen with temperature shift in parental U2OS cells (middle panel). The fold induction of *REDD1* following temperature shift in U2tsp53 cells is unaltered by treatment of cells with 10 μg/ml cycloheximide (Chx), indicating that induction is independent of new protein synthesis (lower panel). Treatment with Chx itself induces an increase in baseline *REDD1* expression. Equal loading was demonstrated for all lanes (data not shown).

(C) Activation of a *REDD1* promoter reporter following transient transfection into U2-tsp53 cells and shift to the permissive temperature (wild-type p53). Schematic representation of the p53-responsive sequence (p53RE, black box), located within a 0.6 kb HindIII/SacI fragment (0.6H/S), upstream of the transcriptional start (arrowhead). The three exons encoding *REDD1* are denoted by white boxes. Activation of this reporter by p53 is abrogated following disruption of the p53RE by four point mutations (0.6-mut). As further control, an adjacent 3.0 kb HindIII promoter fragment (3.0H) demonstrates no induction by p53, nor does vector backbone. Relative luciferase reporter activity is plotted with standard error indicated.

Fraumeni syndrome, induction of *REDD1* by IR was entirely absent, while induction in response to MMS was unchanged (Figure 2A). Identical results were observed with AT960 and AT582, two primary fibroblast lines derived from patients with ataxia telangiectasia (*ATM* null). The induction of *REDD1* following IR is therefore dependent on an intact p53 pathway.

To determine whether ectopic overexpression of p53 alone leads to induction of *REDD1*, a CMV-driven p53 expression construct was transfected into Saos-2 cells, which lack endogenous p53, and into U2OS cells, in which endogenous p53 is destabilized by overexpression of MDM2. In both cell lines, expression of the endogenous *REDD1* transcript was induced following ectopic expression of p53 (Figure 2B). Induction of *REDD1* was also observed in cells with constitutive expression of a temperature-sensitive p53 mutant (U2-tsp53), following growth at the permissive temperature. Since the temperature switch allows activation of wild-type p53 without requiring protein synthesis, we were able to test whether induction of *REDD1* by p53 was independent of new protein synthesis, as expected for a direct transcriptional target. Indeed, while treatment with cycloheximide (Chx) increased baseline expression of *REDD1*, it did not alter the ~5-fold induction of endogenous *REDD1* mRNA by p53 (Figure 2B).

To establish that *REDD1* is a direct p53 transcriptional target, we identified the putative promoter region within the compact (2 kb) *REDD1* genomic locus (Figure 2C). Luciferase reporter assays, using fragments of the *REDD1* promoter as well as intron sequences, mapped a single p53-responsive element to a 0.6 kb HindIII/SacI fragment, immediately upstream of the transcriptional start site. This element was induced ~7-fold following ectopic expression of p53 in Saos-2 cells or temperature switch in U2-tsp53 cells (Figure 2C). Within this element, we identified a single consensus p53 binding site as defined by El-Deiry et al. (1992). Generation of a mutant construct by altering the four critical residues within the core consensus sequence, "Ca/t a/tG", abolished p53-mediated *trans*-activation of the *REDD1* promoter (Figure 2C). Taken together, these observations suggest that *REDD1* is a direct transcriptional target of p53.

REDD1 Is a Member of an Evolutionarily Conserved Gene Family

The *REDD1* open reading frame encodes a predicted protein of 232 amino acids, with orthologs in mouse, *Xenopus*, and *Drosophila* (Figure 3A). The amino acid sequence predicts an acidic, serine-rich protein with strongest evolutionarily sequence conservation at the carboxyl terminus. While several short, conserved helical regions are predicted from secondary structure analysis, no known functional motifs are apparent. A related human transcript is predicted to encode a novel protein with overall 50% identity to REDD1, which we refer to as REDD2. Based on Northern blot analysis of multiple tissue panels, *REDD1* is expressed in most adult tissues, while *REDD2* expression is only detectable by RT-PCR analysis (data not shown). Of note, *REDD1* was also recently identified as *RTP801*, a gene induced in the cellular stress response to hypoxia (Shoshani et al., 2002).

While REDD1 has a predicted molecular weight of 25

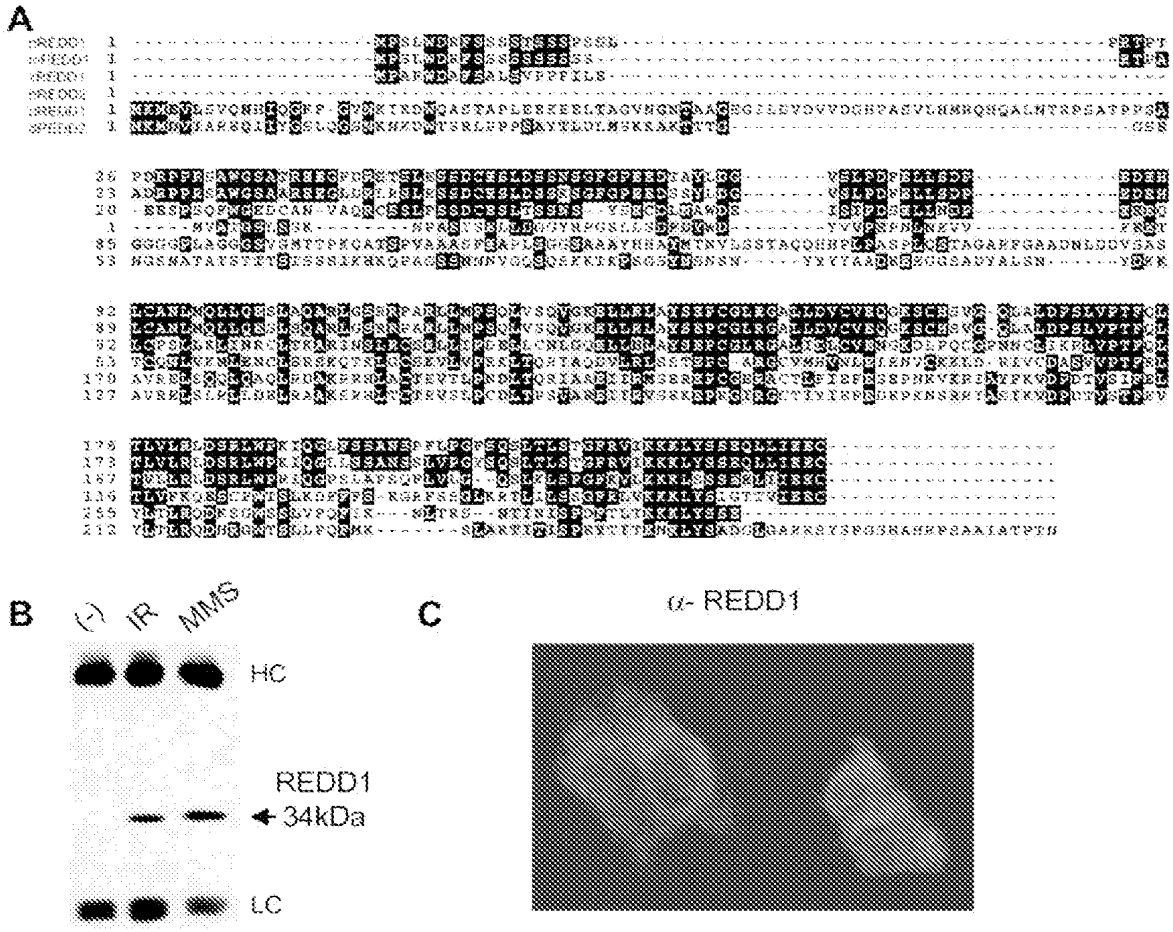

Figure 3. Characterization of the REDD1 Protein

(A) Amino acid sequence alignment of human REDD1 with human REDD2 and with orthologs in mouse (mREDD1), *Xenopus* (xREDD1), and *Drosophila* (dREDD1 and dREDD2, also known as Scylla and Charybde, respectively). Black shading represents identical residues, and gray shading conservative changes.

(B) Immunoprecipitation: Western blot analysis of endogenous REDD1 in U2OS cells, either untreated (−), or 12 hr following 10 Gy IR or 100 μg/ml MMS, using affinity-purified REDD1 polyclonal antiserum. Migration of immunoglobulin heavy-chain (HC) and light-chain (LC) is noted.

(C) Cytoplasmic localization of REDD1 protein in U2OS cells with constitutive expression of a REDD1 construct, as shown by immunofluorescence using affinity-purified REDD1 antiserum.

kDa, the protein migrates at 34 kDa. This was confirmed for endogenous protein recognized by specific polyclonal antisera (Figure 3B), for transiently transfected and in vitro-translated protein products recognized by these antibodies, and for epitope-tagged constructs (data not shown). The aberrant migration is likely to result from multiple lysine residues at the carboxyl terminus. REDD1 protein is present in the cytoplasm, without evidence of localization to specific organelles (Figure 3C), and both endogenous and epitope-tagged REDD1 remain cytoplasmic following DNA damage stimuli (data not shown). In most cells, REDD1 protein is expressed at low levels despite readily detectable mRNA. This most likely results from the short half-life of this protein, estimated to be 15–20 min for transfected REDD1; treatment of cells with proteasome inhibitors stabilizes REDD1, suggesting that the protein is regulated via this degradative pathway (data not shown). Following IR or MMS treatment, endogenous REDD1 protein is increased to detectable levels (Figure 3B). However, this increase in protein expression is correlated with the induction of the *REDD1* message and does not result from any change in the REDD1 protein half-life (data not shown).

REDD1 Expression during Development Parallels that of p63

In striving to generate *REDD1* null mice, we developed mice in which the targeting construct, encoding β-galactosidase (β-gal) linked to the *REDD1* promoter, was integrated in tandem to the endogenous gene. While this did not disrupt endogenous gene expression, it allowed for measurement of *REDD1* transcription during normal development. This in vivo reporter construct included 4 kb of *REDD1* upstream regulatory sequences, the normal start site, first intron, and first splice junction, but replaced the entire *REDD1* coding sequence with β-gal (Figure 4A and Experimental Procedures). Mice expressing a single integrated copy of this reporter were produced. Their embryonic fibroblasts induced β-gal following DNA damage in a pattern consistent with that

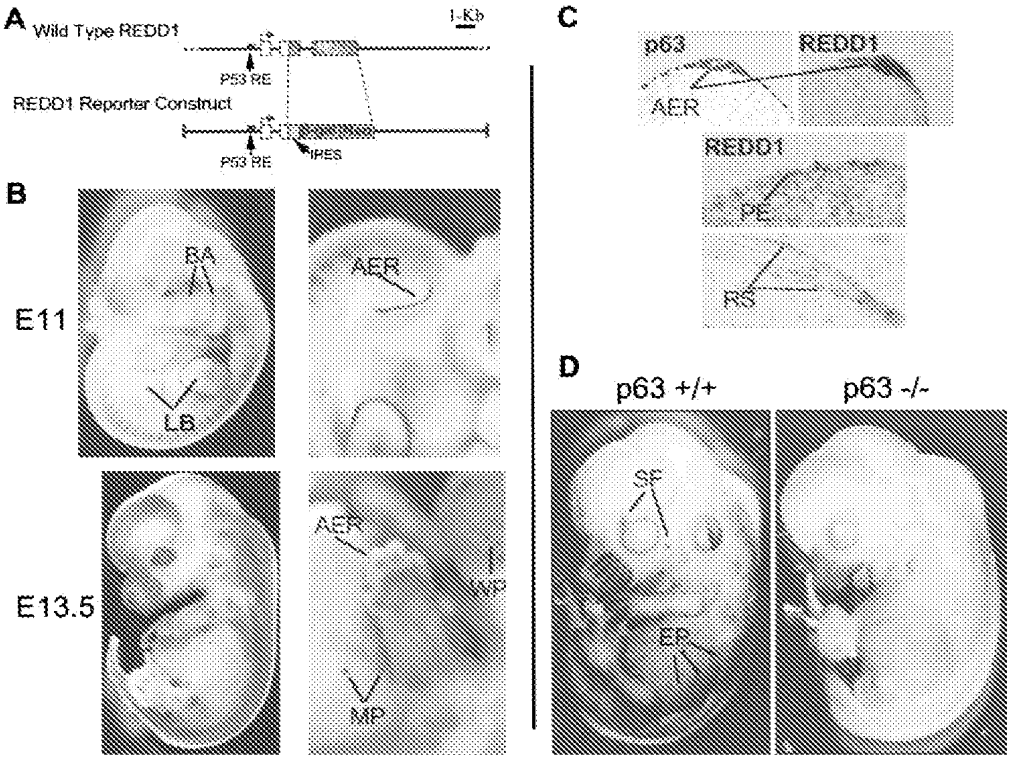

Figure 4. REDD1 Expression Parallels that of p63 during Development

(A) The *REDD1*-β galactosidase (β-gal) promoter reporter construct was synthesized by replacing the coding and 3′ untranslated regions (hatched boxes) with the β-gal/neomycin fusion cDNA. This reporter was inserted by homologous recombination downstream of the endogenous *REDD1* locus (see Experimental Procedures).

(B) β-gal activity in whole-mount embryos carrying one copy of the reporter at embryonic day 11 (E11) or day 13.5 (E13.5). Staining of the apical ectodermal ridge (AER) of the limb bud (LB), the branchial arches (BA), mammary primordia (MP), and developing follicles of the whisker pad (WP) is observed.

(C) Colocalization of p63 protein and REDD1-β-gal within the AER in tissue sections of limb bud from E9.5 embryos (top panels). Like p63, *REDD1* promoter is expressed in cells of the primitive ectoderm (PE; E9.5) and the root sheath (RS; newborn) of the developing hair follicle (lower panels).

(D) RNA in situ hybridization of whole-mount E14.5 wild-type (+/+) or *TP63* null (−/−) embryos, using a *REDD1* antisense probe. Like p63, *REDD1* mRNA expression is normally present at this stage in supraorbital and suborbital follicles (SF), whisker pad, limbs, and patches of developing epidermis (EP) that cover the embryo. These structures, along with *REDD1* expression, are absent in *TP63* null embryos. No staining of wild-type embryos was observed with the control sense *REDD1* probe (data not shown).

demonstrated for endogenous *REDD1* in human fibroblasts, indicating that these regulatory sequences were maintained in the integrated construct (data not shown).

β-gal staining of developing embryos containing the reporter showed a striking tissue-specific pattern, reminiscent of the p53-family member p63. Like p63, the most intense *REDD1* staining in early embryos (E11) is noted in the apical ectodermal ridge (AER), a cluster of primitive ectoderm at the tip of the limb bud critical for induction of limb development (Capdevila and Izpisua Belmonte, 2001) (Figures 4B and 4C). At embryonic day 13.5 (E13.5), *REDD1* promoter activity is present predominantly in ectoderm-derived tissues known to express p63, such as the whisker pad, eyelid, breast primordia, and the developing limb (Figure 4B). Staining in developing cartilage of the limbs, tail, and cranium is also detectable. p63 is also characteristically expressed in the single cell-layer of primitive ectoderm throughout the embryo, and at later stages within the developing epidermis and the root sheath of hair follicles (Mills et al., 1999; Parsa et al., 1999; Yang et al., 1999). Tissue sections from embryos and newborn skin stained for β-gal confirmed that the *REDD1* pattern is essentially identical to that of p63 (Figure 4C).

p63 is required for normal differentiation of ectoderm-derived tissues, and as noted above, *TP63* null mice exhibit failure of skin, limb, and mammary development. We therefore used RNA-in situ hybridization to determine whether *REDD1* expression is altered in *TP63* null mice. Staining for *REDD1* RNA in wild-type littermates confirmed the pattern of *REDD1* expression detected in the β-gal reporter mice. Remarkably, *REDD1* expression is virtually absent in *TP63* null embryos (Figure 4D). Together, these data demonstrate spatial and temporal colocalization of these two genes during embryonic development, consistent with physiological regulation of *REDD1* expression by p63.

Transcriptional Regulation of *REDD1* by p63

Unlike p53, which is regulated by posttranslational modification, p63 appears to be regulated primarily at the transcriptional level. *TP63* mRNA is expressed at high

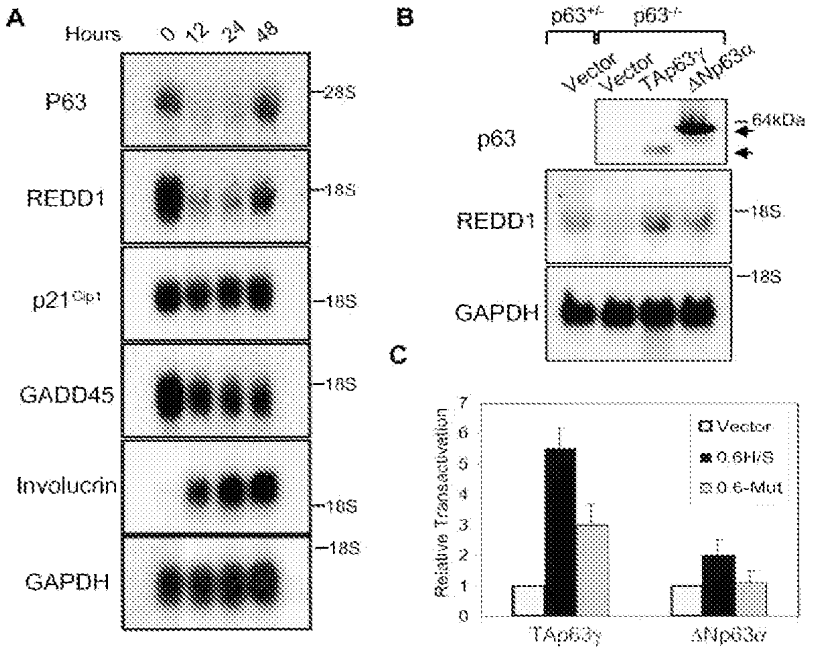

Figure 5. Transcriptional Regulation of REDD1 by p63

(A) Northern blot analysis of *TP63*, *REDD1*, *p21*^{Cip1}, and *GADD45* expression, at sequential intervals following the induction of differentiation in human primary keratinocytes by a change in calcium concentration. Involucrin expression is a marker for keratinocyte differentiation. The same blot was hybridized sequentially with these probes, and *GAPDH* expression is shown as a loading control.

(B) Western blot analysis of lysates from *TP63* null (−/−) mouse embryo fibroblasts (MEFs) infected with the indicated adenoviral constructs and probed with monoclonal anti-p63 antibody (top panel). ΔNp63α (upper arrow) contains an additional C-terminal domain, explaining its slower migration than TAp63γ (lower arrow). Northern blot analysis (lower panels) from either TP63-heterozygous (+/−) or *TP63* null (−/−) MEFs, demonstrating that baseline expression of *REDD1* is reduced in *TP63* null MEFs and restored following adenoviral expression of TAp63γ or ΔNp63α. Note that ~10-fold more ΔNp63α than TAp63γ is required to induce equivalent levels of *REDD1*.

(C) Activation of *REDD1* promoter by both TAp63γ and ΔNp63α isoforms of p63. Promoter reporter constructs encoding the wild-type p53RE (0.6H/S) or mutant sequence (0.6-Mut) were transfected into U20S cells, along with the indicated p63 isoform or vector. The 0.6-Mut fragments lack the 20 bp p53 consensus sequence. Bars show standard error from three independent experiments.

levels in undifferentiated primary keratinocytes and undergoes rapid downregulation following differentiation (Parsa et al., 1999; Pellegrini et al., 2001). To determine whether *REDD1* is coregulated with *TP63* during this process, we examined expression of both genes during in vitro differentiation of human primary keratinocytes. Within hours of differentiation, initiated either by addition of serum or a change in calcium concentration, expression of both *TP63* and *REDD1* transcripts declined markedly, followed by a concordant increase in both transcripts late in differentiation (Figure 5A). In contrast, expression of the p53-regulated genes *GADD45* and *p21*^{Cip1} remained unaltered following differentiation stimuli.

The parallels between *REDD1* and p63 expression in vivo and during induced differentiation suggest positive regulation of *REDD1* by p63. To test this, we first examined *REDD1* expression in mouse embryo fibroblasts (MEFs) derived from *TP63* null mice. *REDD1* mRNA is readily detectable in wild-type and *TP63*-heterozygous MEFs, but is virtually absent in MEFs from *TP63* null mice. Reintroduction of adenoviral TAp63γ restores *REDD1* expression (Figures 5B), directly confirming the ability of p63 to regulate endogenous *REDD1* expression. Of note, REDD1 expression is also induced by the ΔNp63α isoform, which lacks the N-terminal *trans*-activation domain, although much less strongly than by TAp63γ. In contrast to cells lacking p63, expression of *REDD1* is not altered in *TP53* null cells, which however fail to induce *REDD1* transcription following IR. These data are consistent with a model in which p63 predominates in regulating baseline *REDD1* expression, while p53 induces *REDD1* specifically in response to DNA damage stimuli.

Transcriptional regulation of *REDD1* by p63 is expected to depend on the p53 family response element in the *REDD1* promoter. Indeed, cotransfection of reporter constructs containing this promoter sequence and constructs encoding the TAp63γ isoform into U2OS cells demonstrated approximately 5-fold activation, which was largely dependent upon the presence of this consensus p53 response element (Figure 5C). Similar activation was seen following transfection into *TP63* null MEFs (data not shown). The observed *trans*-activation by TAp63γ cannot be attributed to an indirect effect through endogenous p53 since these MEFs were immortalized by expression of SV40 large T antigen (SV40TAg), which inactivates p53. The ΔNp63α isoform demonstrated weak but significant activation of the reporter, consistent with its ability to regulate endogenous REDD1 (Figure 5C). This activity is in keeping with the direct or indirect *trans*-activation potential of ΔNp63α identified in some studies (Dohn et al., 2001), and suggests that transcriptional activation, rather than repression, is the net result of p63 expression.

Regulation of Keratinocyte Differentiation, ROS, and Oxidative Stress Sensitivity by REDD1 and p63

Despite the critical function of p63 during epithelial development, assays to examine its functional properties have not been well defined. The observation that both p63 and REDD1 are rapidly downregulated during the differentiation of human primary keratinocytes prompted us to ask whether suppression of these genes is required for differentiation to occur. We used human adenoviral vectors to express REDD1, TAp63γ, or ΔNp63α in primary human keratinocytes (Figure 6A). Adenoviral expression of these proteins alone did not significantly alter the survival or proliferation of undifferentiated cultures (data not shown). However, constitutive expres-

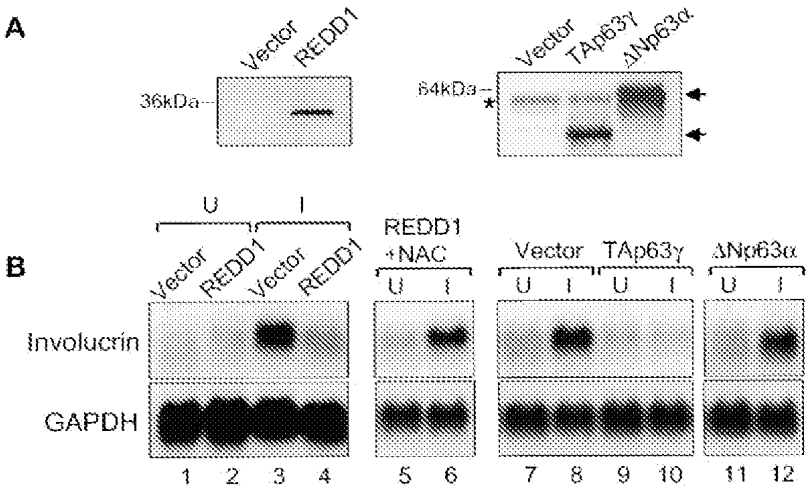

Figure 6. Inhibiton of Keratinocyte Differentiation by REDD1 and p63

(A) Adenoviral expression of REDD1, TAp63γ (lower arrow), and ΔNp63α (upper arrow) in primary human keratinocytes. Lysates from cells infected with the indicated constructs were probed with affinity-purified polyclonal anti-REDD1 antibodies or with monoclonal anti-p63. Endogenous ΔNp63α is detectable in undifferentiated cells (asterisk), and migrates slightly faster than epitope-tagged ectopically expressed ΔNp63α.

(B) Northern blot analysis of primary human keratinocytes infected with the indicated adenoviral constructs. Following infection, cells were left uninduced (U) or were induced (I) to differentiate by calcium shift. Induction of the differentiation marker involucrin (lanes 1 and 3, and 7 and 8) is inhibited by REDD1 (lanes 2 and 4) and TAp63γ (lanes 9 and 10) but not by ΔNp63α (lanes 11 and 12), while addition of the antioxidant NAC abrogates REDD1-mediated inhibition of differentiation (lanes 5 and 6).

sion of REDD1 or TAp63γ nearly completely inhibited induction of keratinocyte differentiation following calcium shift, as measured by induction of the differentiation marker involucrin (Figure 6B). In contrast, expression of ΔNp63α or the adenoviral vector alone had little or no effect on baseline or differentiation-induced involucrin expression (Figure 6B). The inability of ΔNp63α to inhibit cellular differentiation is consistent with its weak *trans*-activational properties, compared with TAp63γ (Figure 5C). These data suggest that p63 and REDD1 expression contribute to preservation of the undifferentiated state, and that their downregulation is required for normal keratinocyte differentiation.

While this work was in progress, *REDD1* was also identified as *RTP801*, a hypoxia-inducible gene involved in the regulation of cellular ROS (Shoshani et al., 2002). Ectopic expression of *RTP801* was shown to modulate cellular ROS levels and sensitivity to oxidative stress. ROS levels are known to be regulated by cellular stress and to contribute to p53-dependent apoptosis (Polyak et al., 1997; Li et al., 1999). However, recent evidence has also implicated ROS as intracellular messengers in receptor signaling pathways linked to cellular proliferation and differentiation (Adler et al., 1999; Finkel, 2000; Meng et al., 2002). To determine whether p63 is involved in regulation of cellular ROS, we first compared ROS levels in *TP63*-heterozygous and *TP63* null MEFs, using the indicator dye CM-H_2DCFDA, whose fluorescence is proportional to the level of multiple forms of intracellular ROS (Ohba et al., 1994). Remarkably, *TP63* null MEFs had significantly lower levels of ROS compared with cells from wild-type or heterozygous littermates (Figure 7A). This difference was consistently observed between fibroblasts derived from multiple embryos, and was detectable in both early- and late-passage MEFs. The difference in ROS was also detectable following transformation of multiple MEF lines by SV40TAg, implying that p63-dependent changes in cellular redox status are not mediated indirectly through modulation of p53 function (Figure 7A). The magnitude of the difference in cellular ROS attributable to p63 is potentially physiologically significant, as it is comparable to the change observed following acute treatment of cells with a lethal dose of hydrogen peroxide (0.8 mM H_2O_2), a major cellular mediator of oxidative stress (Figure 7A). However, H_2O_2 itself is likely to constitute only a subset of the multiple ROS species regulated by p63. Adenoviral-mediated expression of TAp63γ in *TP63* null cells did not significantly change baseline ROS, but it led to a marked enhancement of H_2O_2-induced ROS levels (Figure 7B). Expression of ΔNp63α had little or no effect as compared to the vector control. Adenoviral expression of REDD1 in *TP63* null MEFs enhanced H_2O_2-induced ROS levels to a similar degree as TAp63γ expression (Figure 7B). These data suggest that reduced REDD1 expression in *TP63* null cells contributes to their decreased ROS levels, and that regulation of REDD1 by TAp63γ modulates cellular ROS.

To determine the functional significance of altered ROS levels in *TP63* null cells, we compared the survival of *TP63*-heterozygous or *TP63* null MEFs following treatment with H_2O_2. *TP63* null cells displayed 2-fold enhanced resistance to peroxide challenge, as compared to *TP63*-heterozygous cells (Figure 7C). This difference was observed in both primary and SV40TAg-transformed MEFs, indicating that the difference does not depend on intact p53 function. The resistance to oxidative stress was specific, since *TP63* null cells were not markedly more resistant to DNA damage induced either by the alkylating agent methyl methane sulphonate (MMS) or by ionizing radiation (data not shown), consistent with previous observations (Flores et al., 2002). Adenoviral expression of either REDD1 or TAp63γ in *TP63* null MEFs did not alter baseline cell viability or proliferation (data not shown), but it doubled the number of cells killed following H_2O_2 challenge (Figure 7C). This effect was specifically linked to the increase in ROS associated with expression of REDD1 or TAp63γ, since treatment of cells with the antioxidant N-acetyl cysteine (NAC) blocked induction of ROS and effectively suppressed the increased cell killing observed in p63 and REDD1-reconstituted cells exposed to H_2O_2 (Figures 7B and 7C).

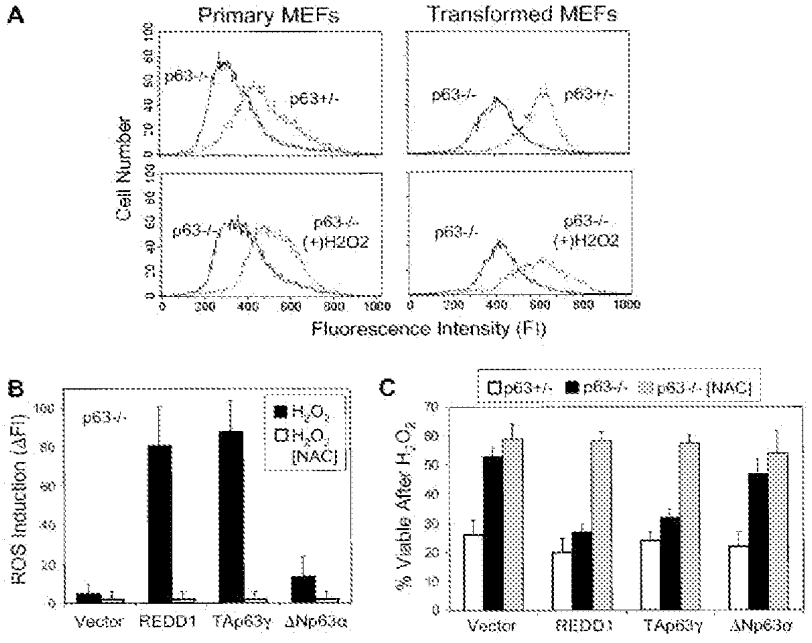

Figure 7. Regulation of ROS and Oxidative Stress Sensitivty by p63 and REDD1

(A) Levels of intracellular ROS in *TP63*-heterozygous (+/−) and *TP63* null (−/−) primary and transformed MEFs, as assessed by the fluorescent indicator dye CM-H_2DCFDA. The difference in baseline ROS between *TP63*-heterozygous and null MEFs is comparable in magnitude to the ROS induced following acute treatment with 0.8 mM H_2O_2 (lower panels). Similar differences in ROS levels were observed between wild-type and *TP-63* null MEFs (data not shown). Cellular transformation alters baseline ROS levels but does not change the difference in ROS observed between *TP63*-heterozygous and null cells.

(B) Induction of ROS following peroxide challenge. *TP63* null MEFs were infected with the indicated adenoviral constructs, followed by treatment with 0.4 mM H_2O_2 for 12 hr in the presence or absence of 10 mM NAC. ROS induction was quantitated by CM-H_2DCFDA fluorescence (increase in mean fluorescence intensity). Expression of either REDD1 or TAp63γ markedly enhances ROS induction, which is blocked by NAC. Error bars denote standard error for three independent experiments.

(C) Cellular sensitivity to oxidative stress. *TP63*-heterozygous or null MEFs were infected with the adenoviral constructs indicated, then treated with 0.8 mM H_2O_2 in the presence or absence of 10 mM NAC for 24 hr. Percent viable cells is shown compared to cultures without H_2O_2 treatment. *TP63* null MEFs are resistant to oxidative stress, and sensitivity is restored following expression of either REDD1 or TAp63γ; NAC abrogates increased oxidative stress sensitivity mediated by REDD1 or TAp63γ. Error bars denote standard error for at least three independent experiments.

NAC did not further suppress oxidative stress sensitivity of vector-infected *TP63* null cells (Figure 7C). Remarkably, treatment of primary keratinocytes with NAC completely abolished the inhibition of differentiation mediated by REDD1 (Figure 6B). NAC itself had no effect on calcium-induced keratinocyte differentiation (data not shown). These observations suggest that the enhancement of cellular ROS by REDD1 contributes to p63-mediated inhibition of keratinocyte differentiation.

Discussion

REDD1 was identified here by expression profile analysis as a transcript that is dramatically upregulated following telomere disruption. This finding is consistent with the emerging parallels between pathways involved in telomere maintenance and response to DNA damage (Blackburn, 2000). In this setting, *REDD1* is activated to a greater extent than other well-established DNA damage response genes, $p21^{Cip1}$ and *GADD45*, and it exhibits a distinct induction profile in response to multiple DNA damage stimuli. Induction of endogenous *REDD1* following IR is dependent upon the presence of ATM and p53, placing it downstream of p53 within this well-characterized DNA damage response pathway. Like $p21^{Cip1}$ and *GADD45*, *REDD1* is induced both by p53-dependent and -independent pathways following DNA damage (Loignon et al., 1997; Jin et al., 2001). Consistent with recent observations that p63 participates in p53-mediated DNA damage responses (Flores et al., 2002), *TP63* null cells show decreased *REDD1* induction after ionizing radiation, but not MMS treatment (data not shown). Characterization of additional elements within the *REDD1* promoter that mediate its particular DNA damage-induced transcriptional profile will require further analysis. In addition to the consensus p53 binding sequence, the proximal promoter of *REDD1* contains a functional heat shock element (HSE) (data not shown), and *REDD1* was also recently identified as *RTP801*, a gene induced by the hypoxia-inducible factor HIF-1 (Shoshani et al., 2002). These observations suggest that *REDD1* may be induced in response to multiple cellular stresses.

Transcriptional targets of p53 identified to date are thought to mediate several DNA damage-regulated processes, including cell cycle arrest, apoptosis, and regulation of p53 turnover. p53 is known to induce a number of genes implicated in cellular redox control, which ultimately contribute to p53-mediated apoptosis via the mitochondrial apoptotic cascade (Polyak et al., 1997; Li et al., 1999). The direct transcriptional induction of *REDD1* by p53 underscores the existence of p53-dependent pathways that regulate ROS. Expression of REDD1 alone is insufficient to induce apoptosis in fibroblasts, but reduced REDD1 levels are associated with resistance to oxidative stress, while REDD1 expression increases cellular sensitivity to lethal oxidative stress. In addition, expression of RTP801 [REDD1] may be sufficient to induce apoptosis in some cellular contexts (Shoshani et al., 2002). Determining the precise contribution of REDD1 to p53-dependent apoptotic functions will require studies in cells with targeted inactivation, since the high levels of *REDD1* transcript induced by DNA damage have prevented efficient suppression using RNA interference strategies (data not shown).

Unlike p53, the functional pathways regulated by p63

and its native transcriptional targets are poorly understood. The striking colocalization of *REDD1* expression with p63 in the apical ectodermal ridge and in developing epithelial structures first pointed to a potential regulatory interaction. The highly restricted expression pattern shared by these two genes is also evident at the single-cell level, with both present in the primitive ectoderm during midgestation, and later in the maturing epidermis and hair follicles (Parsa et al., 1999). Underscoring this similarity, *REDD1* is virtually undetectable in *TP63* null mouse embryos. The reduced REDD1 levels in these embryos may result directly from loss of p63-mediated transcription, or may be an indirect consequence of epithelial differentiation abnormalities. Evidence supporting direct transcriptional activation of *REDD1* by p63 includes its *trans*-activation of the *REDD1* promoter, coregulation of the two endogenous transcripts during in vitro differentiation of primary keratinocytes, and most significantly, restoration of baseline REDD1 expression in *TP63* null MEFs following adenoviral reconstitution of p63 expression. Of note, neither the dramatically decreased expression of *REDD1* in *TP63* null MEFs nor the biphasic expression pattern during in vitro keratinocyte differentiation are observed with other p53-induced genes such as $p21^{Cip1}$ and *GADD45*. Regulation of *REDD1* by p63 therefore appears unique in defining a physiological interaction during epithelial differentiation.

The transcriptional program activated by p63 is likely to underlie its regulation of epithelial differentiation. The phenotype of *TP63* null mice has been explained either as a failure of basal stem cells to undergo differentiation or as an initial wave of differentiation coupled with failure of maintenance and renewal of basal stem cells, leading to depletion of the basal stem cell pool (Mills et al., 1999; Yang et al., 1999). The latter hypothesis is based on the presence of differentiated epithelial structures in early embryos from these mice, followed by disappearance of the basal cell layer. While our studies of REDD1 do not distinguish between these possibilities, the coexpression of p63 and REDD1 in the undifferentiated basal cell layer and their ability to inhibit keratinocyte differentiation point to a role in preserving undifferentiated basal cells. The mechanism underlying this effect is likely to be complex, but our observations suggest a role for regulation of reactive oxygen species.

We have found that *TP63* null cells have significantly decreased levels of ROS and reduced sensitivity to oxidative stress, while ectopic expression of either TAp63γ or REDD1 enhances ROS induction and restores sensitivity. In contrast, ΔNp63α did not regulate ROS or oxidative stress sensitivity, which may in part reflect its relatively weak ability to induce REDD1. Taken together, these studies implicate REDD1 as a mediator of p63-dependent redox regulation, although the mechanism by which REDD1 modulates intracellular ROS is unknown. Direct generation of ROS by REDD1 seems unlikely since the REDD1 protein does not possess homology to known cellular oxidative enzymes, and it does not appear to localize to sites of ROS production within mitochondria. Further insights may be derived from characterization of the specific reactive species generated by REDD1 since treatment of keratinocytes by H_2O_2 alone does not replicate the effect of REDD1 on cellular differentiation (data not shown). The identification of *REDD1*-*RTP801* as a downstream target of both p53 and HIF-1 suggests that it may in fact function in concert with other redox-regulatory genes known to be induced by these two transcription factors.

The finding that loss of p63 alters cellular ROS levels may provide one mechanism by which p63 modulates differentiation and interacts functionally with other factors involved in epithelial development. In addition to effects on cellular stress and viability, subtle shifts in intracellular ROS levels have recently been shown to modulate cellular signaling through multiple tyrosine kinase growth factor receptors, including the epidermal growth factor receptor (EGF-R) (Bae et al., 1997; Finkel, 2000; Meng et al., 2002). Regulation of EGF-dependent responses by ROS may therefore contribute to the effect of p63 on keratinocyte differentiation in vitro, as well as the failure of epidermal differentiation in *TP63* null mice. In this regard, another growth factor, fibroblast growth factor-8 (FGF-8), is of particular interest since it is specifically coexpressed with p63 and REDD1 in the AER and is critical for induction of limb development (Capdevila and Izpisua Belmonte, 2001). Taken together, our data raise the possibility that regulation of ROS levels by p63 plays a role in its modulation of epithelial differentiation.

Experimental Procedures

Cell Lines and Cell Culture

Saos-2 and U2OS are human osteosarcoma-derived cell lines. U2tsp53 is a U2OS cell line expressing the temperature-sensitive p53val135 mutant. Human primary diploid fibroblasts and MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% fetal bovine calf serum (FBS). Primary human epidermal foreskin keratinocytes (HFK) were cultured in keratinocyte serum-free medium (GIBCO-BRL) containing epidermal growth factor (EGF), bovine pituitary extract (BPE), and 0.4 mM calcium as described (Dickson et al., 2000 and references therein). HFK differentiation was initiated by changing the calcium concentration of the growth media from 0.4 to 0.05 mM. U2OS-derived cell lines expressing inducible hTERT or hTERT-DA were created using the "tet-off" system (Clontech).

Oligonucleotide Array-Base Expression Profiling

Gene expression profiles in inducible hTERT-DA cells were analyzed using Affymetrix Gene Chip microarrays as previously described (Ellisen et al., 2001b). Total RNA was isolated 12 and 24 hr after tetracycline washout and was subjected to reverse transcription, labeling, and hybridization as previously described (Lockhart et al., 1996). A 5-fold cutoff was used to define significant message induction by microarray, and only genes whose induction was verified by Northern blot were analyzed further.

Cloning and Analysis of the REDD1 Promoter

The human REDD1 genomic locus was isolated from a BAC library (Research Genetics). Restriction enzyme fragments flanking and including the REDD1-transcribed sequence were subcloned into the pGL-3 basic vector and screened for responsiveness to p53 or p63, as indicated using the Dual-Luciferase assay system (Promega). Secreted HGH or *Renilla* luciferase expression (pRL-TK, Promega) was used as a transfection control. The mutant 0.6-mut and 0.6-Mut fragments were created by standard PCR-based site-directed mutagenesis procedures.

Targeted Incorporation of the REDD1 In Vivo Reporter

A 14 kb mouse genomic fragment containing the *REDD1* transcription unit was isolated from a Lamda Zap II bacteriophage library (Stratagene). To generate the targeting vector, the entire *REDD1* coding region was excised and replaced with an IRES linked to the β-gal/neomycin-resistance fusion gene such that transcription is

driven by the endogenous *REDD1* promoter. ES cell clones were screened for the presence of the targeting construct at the endogenous *REDD1* locus, and were found to have incorporated the construct immediately downstream of the intact REDD1 transcription unit. Animals carrying a single germline copy of the inserted reporter were created using standard procedures (Hogan et al., 1994).

β-Gal Staining and In Situ Hybridization of Whole-Mount and Sectioned Embryos

Whole-mount and frozen section staining for β-gal was carried out as described (Hogan et al., 1994). Immunohistochemical staining for p63 was performed using the 4A4 anti-p63 antibody as described (Yang et al., 1999). Whole-mount in situ hybridization was performed essentially as described (Wilkinson, 1992), using digoxigenin-labeled antisense and sense (control) mouse *REDD1* RNA probes. All embryos and sections were viewed by standard light microscopy.

Quantitation of Cellular ROS and Oxidative Stress Sensitivity

Intracellular levels of ROS were assayed using the fluorescent indicator dichlorofluorescein diacetate (CM-H_2DCFDA, Molecular Probes, Inc.). Cells were stained with 1 μg/ml of the indicator in serum-free medium for 30 min, then washed, trypsinized, and analyzed immediately. Analyses were carried out using the FACSCaliber flow cytometer and Cell Quest software (Becton Dickinson). For viability assays, following adenoviral infection, subconfluent cultures were left untreated or were treated with H_2O_2 for 24 hr, then cells were stained with 0.2% trypan blue, and viable cells were counted in triplicate for each data point. Where indicated, cells were pretreated with NAC for 2 hr prior to addition of H_2O_2.

Adenoviral-Based Gene Expression

REDD1, TAp63, and ΔNp63 cDNAs were subcloned into the pAdShuttle-CMV vector and were cotransformed with the pAdEasy-1 plasmid into recombination-proficient BJ5183 cells (all from Stratagene). The γ splice variant of the TAp63 isoform and the α variant of ΔNp63 were used in these constructs, since they are the best characterized species. Resulting recombinants were transfected into 293 cells for viral production. Adenoviral stocks were titered using standard viral plaque assays and were used at an MOI of 50 for all infections, except as noted (Figure 5B). All cellular assays were initiated 24 hr following adenoviral infection.

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